

CALCIUM ACTIVATED NEUTRAL PROTEASE FROM HUMAN SKELETAL MUSCLE

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1. Introduction

Various proteases are involved in the myofibrillar protein degradation, although the nature of the proteases and its mechanism are not clear yet. Since Ca^{2+} activated neutral protease (CANP) specifically removes the Z-band, which is important in maintaining the myofibrillar structure, CANP is thought to play an important role in the initial step of myofibrillar protein degradation [1]. CANP was first identified by Huston and Krebs [2] as a kinase-activating factor and was later partially purified from pig muscle by Dayton et al. [3,4]. They reported that CANP is composed of two subunits of molecular weights of 80 000 and 30 000 [3,4]. Recently we purified CANP for the first time from chicken muscle and found that the protease is a monomer of molecular weight of 80 000 [5]. As the pig enzyme is not completely pure, it is not clear whether the protein of molecular weight of 30 000 is a contaminant or not. In order to resolve the apparent discrepancy in the subunit composition of CANP, we tried to isolate CANP from a mammalian source, human skeletal muscle. In the present paper isolation of human muscle CANP is reported together with its subunit composition and some other properties.

2. Materials and methods

Skeletal muscle obtained from human cadaver free from neuromuscular diseases was stored at -20°C until use. Chicken CANP and other materials

used are the same as described before [5]. The CANP activity was measured by the method described previously [5]. The antiCANP serum antibody was prepared by injecting 1 mg of purified CANP with Freund's adjuvant into the rat foot pads 2 times at 2 weeks interval [6].

3. Results

3.1. Purification of human CANP

The method described here is similar to that applied for chicken muscle CANP [5]. All procedures were performed at $0-4^{\circ}\text{C}$. Frozen muscle (813 g) was sliced and homogenized in a Waring blender with 2 volumes of 20 mM Na_2CO_3 —1 mM EDTA for 5 min. The pH of the crude extract obtained by centrifugation was adjusted to 4.9 by addition of 1 N acetic acid and the precipitate was collected after 30 min. The precipitate was extracted overnight under stirring with 100 ml of buffer A (20 mM Tris—HCl—0.1 M NaCl—5 mM EDTA—10 mM 2-mercaptoethanol, pH 7.0). The extract was applied to a DEAE-cellulose column (DE-52, 4.5×40 cm) equilibrated with buffer A. The column was developed with a linear NaCl gradient from 0.1 M to 0.8 M in a total volume of 3 litres of buffer A. CANP eluted at a NaCl concentration of ca. 0.38 M was pooled and precipitated by addition of solid ammonium sulfate up to 60% saturation (at 25°C). The precipitate was dissolved in 3 ml of buffer A' (same as A but pH 7.5) and chromatographed on an Ultrogel AcA 34 column (1.5×145 cm). The active fractions were pooled and

further purified by a column of DEAE-Sephadex A-50 (0.9 × 45 cm). CANP was eluted from the column with a linear gradient of 0.1 M to 0.8 M NaCl in a total volume of 300 ml of buffer A'. The active fractions were stored at 4°C. The results of purification of human muscle CANP are summarized in table 1.

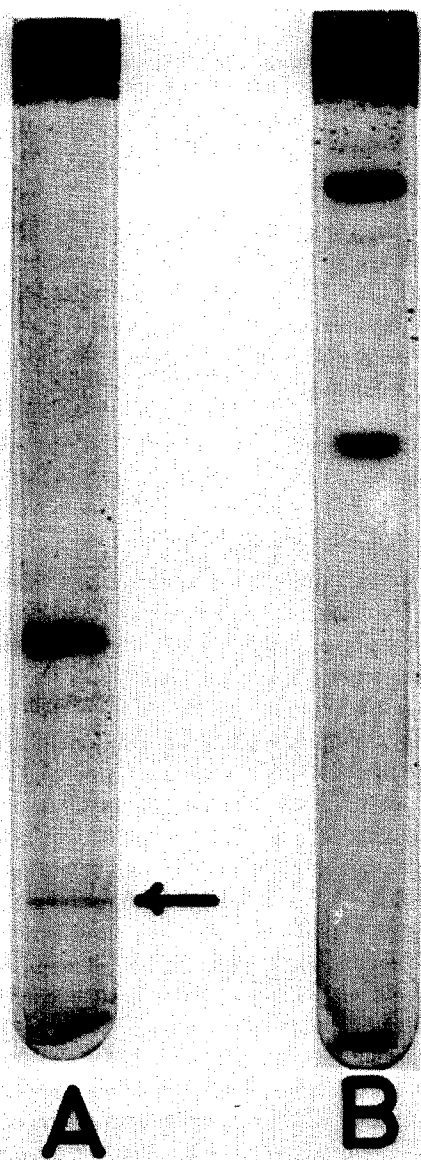


Fig.1. Polyacrylamide gel electrophoresis of purified human muscle CANP in the absence (A) and presence (B) of SDS. Arrow indicates the position of maker dye.

3.2. Properties

Human muscle CANP thus purified was homogeneous as judged by disc gel electrophoresis (fig.1A) and by electrophoresis on cellulose acetate strips at various pH values. The protease gave two bands upon SDS-gel electrophoresis (fig.1B). The molecular weights of these two bands were estimated to be 78 000 (80 k) and 28 000 (30 k), and their amount ratio was 1.0 : 1.0–1.2 by tracing the gel. The molecular weight of the protease determined by Ultrogel filtration using various marker proteins was 100 000–110 000. Thus human muscle CANP is a dimer composed of two different subunits of molecular weights of 78 000 and 28 000. The molecular weight of 80 k subunit was identical to that of chicken muscle CANP by SDS gel electrophoresis. The specific activity of human muscle CANP was about 340 unit/mg and was almost comparable to that of chicken muscle CANP (300 unit/mg).

Human CANP was activated by Ca^{2+} ions ($pK = 2.8 \text{ mM}$) as shown in fig.2. The concentration of Ca^{2+} ions was slightly higher than that for chicken muscle CANP ($pK = 0.9 \text{ mM}$) examined under the same conditions. Various divalent metal ions (Sr^{2+} , Ba^{2+} , Mg^{2+} , Co^{2+} , Ni^{2+} , etc.) did not activate human muscle CANP at concentrations up to 10 mM,

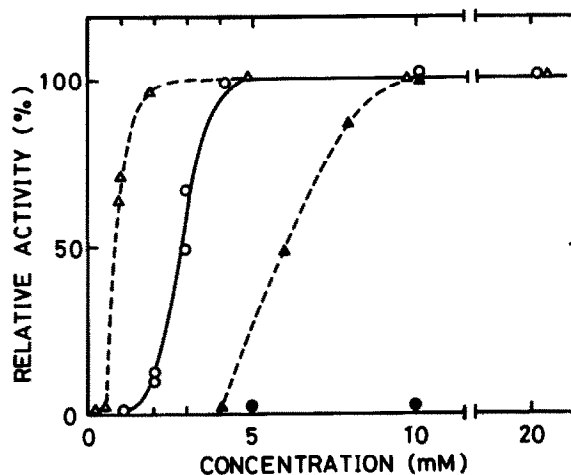


Fig.2. Activation of human muscle CANP by Ca^{2+} ions. The activity was measured in the presence of various concentration of Ca^{2+} (○) and Sr^{2+} (●) ions. The result of chicken muscle CANP was also indicated as a control (△ and ▲ for Ca^{2+} and Sr^{2+} , respectively).

Table 1
Purification of CANP from human skeletal muscle (813 g)

Fraction	Protein (mg)	Specific activity (u/mg)	Recovery (%)
Crude extract	56 100	0.024	100
Extract of acid precipitate	898	2.54	182
DEAE-cellulose fraction	76.0	27.8	169
Ultrogel fraction	14.2	91.8	104
DEAE-Sephadex fraction	1.7	340	47

although chicken CANP was fully activated by 10 mM Sr^{2+} ions.

The optimum pH of human CANP was 7.5 in Tris-HCl and glycerophosphate-HCl buffers, while chicken CANP was optimally active at pH 7.8. Therefore CANP expresses its full activity at a physiological pH region. The isoelectric pH values of human and chicken CANPs were estimated to be 4.5 and 4.9, respectively, by electrophoretic mobilities on cellulose acetate strips [7].

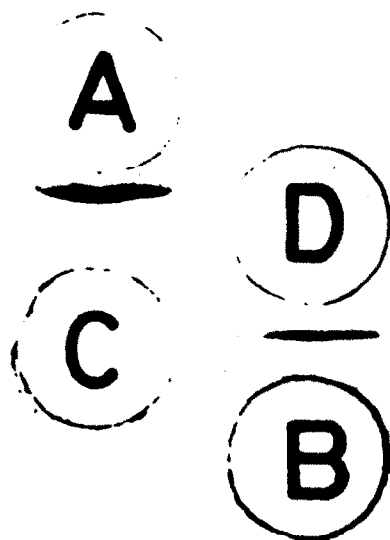


Fig.3. Ouchterlony double diffusion analysis of purified human muscle CANP. A, B: purified chicken and human muscle CANPs, respectively. C, D: antibody to chicken and rabbit muscle CANPs, respectively.

3.3. Inhibitor of human CANP

Various compounds were added to the assay mixture and their effect on the activity of human CANP was examined. Leupeptin, antipain, and chymostatin strongly inhibited the enzyme activity. The molar ratios of inhibitors to CANP at 50% inhibition were 25, 52, and 100, respectively, for human CANP, while those for chicken CANP were 15, 55, and 150, respectively. Both proteases were not strongly inhibited by pepstatin. The activity was not affected by soybean trypsin inhibitor and phenyl methane sulfonyl fluoride, but was completely destroyed by iodoacetic acid and *p*-chloromercuribenzoate (concentration: 100 M excess). These results are quite similar to those obtained with chicken CANP and indicate that human CANP is probably an SH- rather than a serine-enzyme.

3.4. Immunological test

As shown in fig.3 human CANP did not cross-react with the antibody against chicken CANP but gave a single precipitin line with the antibody against the monkey enzyme which is composed of two subunits of 80 k and 30 k (unpublished results). While chicken CANP did not give a precipitin line with the antibody against the monkey enzyme, though it gave a single line with the chicken muscle CANP-antibody. Hence human muscle CANP is immunologically different from the chicken enzyme but is similar to the monkey enzyme which has the same subunit composition as human CANP.

4. Discussion

The result that human CANP is composed of two subunits of 80 k and 30 k is consistent with that reported by Dayton et al. [3,4] for the pig muscle enzyme, although the latter is not completely pure. We also purified rabbit and monkey muscle enzymes and found that both enzymes are composed of two subunits of 80 k and 30 k (to be published). Hence all mammalian muscle CANPs thus far purified are dimers of two different subunits. Whereas chicken muscle CANP is a monomer and is clearly different from mammalian CANP as far as the subunit composition is concerned. The fact is partly supported by the immunological test shown in fig.3. The properties of human muscle CANP except for the subunit composition are, however, very similar to those of chicken muscle CANP. It is therefore quite interesting to examine the role of the 30 k subunit which is found only in mammalian muscle CANPs. It should be noted that as for CANP from mammalian non muscle sources, molecular weights of 93 000 and 115 000 are reported for the enzymes from rat brain [8] and liver [9], and calf uterus [10], respectively. Recently CANP was also found in human platelets [11]. Although partially purified enzymes were used in these studies, the results indicate the necessity of further comparative studies of CANP.

References

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